EXHIBIT B

Epithelial Transport of Drugs in Cell Culture. I: A Model for Studying the Passive Diffusion of Drugs over Intestinal Absorbtive (Caco-2) Cells

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Abstract □ A human intestinal cell line, Caco-2, was used as a model to study the passive diffusion of drugs across intestinal epithelium. The cells formed continuous monolayers when grown on permeable filters of polycarbonate. After 10 days in culture, the monolayers had a transmembrane resistance of ~260 ohms·cm^2 and a cell density of 0.9×10^5 cells/cm^2. At this time the cells were impermeable to [^{14}C]polyethyleneglycol (MW 4000). These characteristics remained constant for 20 days (i.e., from day 10 to day 30). Six beta-blocking agents with a 2000-fold range of lipophilicity were studied for their transepithelial transport properties. The transport parameters were independent of drug concentration and transport direction. The apparent permeability coefficients ranged from $41.91 \pm 4.31 \times 10^{-6}$ cm/s for the most lipophilic drug, propranolol, to $0.203 \pm 0.004 \times 10^{-6}$ cm/s for the most hydrophilic drug, atenolol. The transport parameters were compared with those published for rat ileum. The transport rates were similar for four out of five drugs. Atenolol was transported at a slower rate in the Caco-2 model, which may be explained by the fact that the Caco-2 cells form a tighter epithelium than the rat ileal enterocytes. The findings of this paper indicate that Caco-2 cells may be used to model the intestinal absorption of drugs.

The determination of oral absorption is an important part in the preformulation of new drug entities. Consequently, studies on intestinal absorption of drugs have received considerable attention.[1,2] In general, two types of models are available for absorption studies. The animal models are based on the in situ isolation of intestinal loops. The drug is administered into the loop and the disappearance rate from the loop and/or appearance in the blood is measured.[3] In the alternative in vitro models, an intestinal segment is isolated and mounted in an Ussing chamber.[4] In this case, the intestinal segment is used as a semipermeable membrane between a donor and a receiver chamber. The development of these models has made it possible to characterize several factors that determine the transepithelial transport of drugs.[1]

Although the available models have been used frequently over the years, they have some drawbacks. First, they are not of human origin. Second, they are relatively complicated and consequently only a limited number of experiments can be performed on each occasion. Third, the duration of the experiments are often limited to from a few minutes[2] to a few hours.[3] A recent in vivo model, which allows the chronic in situ isolation of an intestinal loop in rats, may provide a solution to this problem.[3] However, gradual disintegration of intestinal segments in the in vitro models is still a problem.[2] Thus, there is a demand for new and better absorption models.

One approach to a simple and reproducible absorption model is to cultivate intestinal cells on permeable membranes.[5-7] In such a model a larger number of experiments can be performed simultaneously and over relatively long time periods. Moreover, sampling on the luminal and basolateral side of the epithelium is possible and crossover studies can be performed. However, until recently, this approach has not been possible due to the lack of differentiated intestinal cell lines.[6] The recent availability of a well-differentiated human intestinal cell line, Caco-2, now makes it possible to investigate the possibility of using monolayers of intestinal cells for studies of passive drug absorption.

Caco-2 monolayers have recently been used to investigate the transport of ions and different endogenous products over the intestinal epithelium (reviewed in ref 6). Thus, the active transport of bile acids,[7,9] vitamins,[7,10] and amino acids[7,11] has been studied. It is likely that the Caco-2 model will be useful in studies of actively transported drugs. However, with few exceptions, drugs are transported over the intestinal epithelium by passive diffusion.[1] In this study, the Caco-2 model was investigated for its usefulness in studying the passive absorption of drugs. Apparent permeability coefficients (P_{app}) for a homologous series of β-blockers are determined and compared with published in vivo data.

β-Adrenoceptor antagonists were chosen as model drugs for several reasons. They represent a homologous series of clinically relevant drugs and, additionally, they have similar pK_a values (~9.5) and molecular weights (~260) and cover a relatively broad range of different lipid solubilities. Thus, one of the most important factors that determines drug absorption, lipophilicity,[1] could be studied. Moreover, absorption data from experimental animals were available for all except one of the β-blocking agents. Finally, there are no data in the literature that indicate that these drugs are significantly metabolized by the intestinal epithelium.[12]

Experimental Section

Drugs and Radiolabeled Markers—^{3}H-Labeled and unlabeled atenolol (specific radioactivity 0.627 nCi/nmol), H216/44 [(*S*)-4-hydroxy-*N*-(2-((2-hydroxy-3-(4-(2-(2-(cyclopropylmethoxy)ethoxy)ethyl)phenoxy)-propyl)amino)ethyl)-1-piperidinecarboxamide; specific radioactivity 16.7 μCi/nmol], alprenolol (specific radioactivity 0.252 nCi/nmol), and metoprolol (specific radioactivity 0.475 μCi/nmol, as well as ^{14}C-labeled practolol (2.1 nCi/nmol) were generous gifts from Dr. Kurt-Jörgen Hoffman, AB Hässle, Göteborg, Sweden. [^{3}H]Propranolol (26.6 Ci/mmol) and [^{14}C]polyethyleneglycol (MW 4000; specific radioactivity 15.0 Ci/mg) were obtained from New England Nuclear, Boston, MA. The radiolabeled compounds had a radiochemical purity of 96–99%, Propranolol and alprenolol were purchased from Sigma Chemical Company, St. Louis, MO.

Octanol–water distribution coefficients (D) and pK_a values for the β-blockers were kindly provided by Dr. Kurt-Jörgen Hoffman, AB Hässle, Göteborg, Sweden, and have also been published elsewhere.[12-14]

Cells—Caco-2 cells, originating from a human colorectal carcinoma,[15] were obtained from American Tissue Culture Collection, Rockville, MD. The cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum, 1% nonessential amino acids, benzylpenicillin (100 U/mL), and streptomycin (10 μg/mL). Cells of passage number 85–95 were used. All tissue culture media were obtained from Gibco through Laboratorie Design AB, Lindingö, Sweden. The medium was changed every second day.

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The cells were mycoplasma negative as determined with Hoechst 33258.[16]

The cells were expanded in tissue culture flasks (225-cm² growth area). The cells were detached from the flasks by treatment with trypsin (0.25% in phosphate-buffered saline; PBS) containing 0.2% EDTA. The cells were added to polycarbonate membrane culture plate inserts (Nucell; pore size 0.4 μm; diameter 24 mm; Nuclepore, Pleasanton, CA) or to inserts made of mixed cellulose esters (Millicell-HA; pore size 0.45 μm; diameter 27 mm; Millipore, Bedford, MA) as described previously.[5,7] The inserts were placed in tissue culture wells (Costar, Badhoevedorp, The Netherlands; cat. no. 3506) and 2 × 10^6 cells were added to each insert (see Figure 1). The cells were allowed to grow and differentiate for up to 30 days. After this time, Caco-2 monolayers may start to detach from their support.[6]

Integrity of the Monolayers—The integrity of the monolayers was determined by measurement of the potential difference over the cells and by following the transepithelial transport of a macromolecular marker, polyethylene glycol (MW 4000). The potential difference was measured as previously described[17] and expressed as transmembrane resistance (ohms·cm²) after subtraction of the intrinsic resistance of the model (i.e., the resistance obtained over cell-free inserts). The intrinsic resistance was obtained by solubilization of the cells with 100 mM sodium taurocholate (Sigma Chemical Company, St. Louis, MO) for 15 min at room temperature. The resistance of the resulting cell-free inserts varied with the age of the electrodes, but generally did not exceed 70 ohms·cm². Identical results were obtained with empty filters.

[^{14}C]Polyethyleneglycol$_{4000}$ (16.7–66 μg) was added to the apical side of the monolayers and the transport of the radiolabeled marker was followed for up to 6 h at 37 °C. After 1, 2, 3, 4, and 6 h, 100 μL (out of 2000 μL) was withdrawn from the apical and basolateral chambers and the corresponding volume of fresh medium was added. The samples were measured in a liquid scintillation counter. Inserts without cells were used to determine the maximal transport of the marker during the same time period. The results were expressed as percentage transported of the dose.

Determination of Cell Density—The cell density was determined from photomicrographs by counting the number of hematoxylin-stained cell nuclei on the transparent filters. The cell-covered filters were removed from the inserts and washed twice with PBS (pH 7.4). A chilled 3% solution of freshly prepared glutaraldehyde (4 °C) in PBS was added. After 15 min at room temperature, the inserts were washed with PBS and stored in PBS at 4 °C until further use. The glutaraldehyde-fixed cells were stained with hematoxylin using standard procedures. The monolayers were transferred to ethanol through a concentration gradient of 50, 70, 90, and finally 100% ethanol. The filters were made transparent by treatment with xylene.[7] The filters were mounted on microscope slides (DPX mountant; BDH, Ltd., Poole, U.K.). The photomicrographs were obtained in an inverted light microscope (Olympus CK 2) using a ×20 objective and ×3.3 photo eye piece. At least 1000 cells were counted in each sample.

Measurements of Drug Transport—Drug solutions were prepared from the radiolabeled isotopes and the corresponding unlabeled compounds in ethanol to give final concentrations of 1 × 10^{-3}, 1 × 10^{-4}, or 1 × 10^{-4} M. The ethanol concentration in the cell culture medium was usually ~0.05% and never exceeded 0.25%. Control experiments, including measurements of transmembrane resistance, [^{14}C]PEG transport, light microscopy, and transmission electron microscopy showed that the monolayers were unaffected by this concentration of ethanol.

All transport experiments were performed in air at 95% relative humidity and 37 °C in serum-free medium (DMEM; pH 7.3) containing 1% nonessential amino acids, 10 mM HEPES buffer, and 0.1% human serum albumin. Under these conditions, the integrity of the monolayers was intact for at least 6 h. The monolayers were agitated on a microscope slide mixer (Relax 3, Kebo Lab, Stockholm, Sweden) at 10 rpm and a 2.5 ° angle. The radiolabeled drug solutions were usually added to the apical chamber and the samples were mixed for 30 s. At this time, the initial concentration (C_o) in the donor chamber was determined from a 50- or 100-μL sample. (No radioactivity was found in the receiving chamber after 30 s.) Subsequently, a maximum of five samples (50–100 μL) were taken from each chamber at regular time intervals).

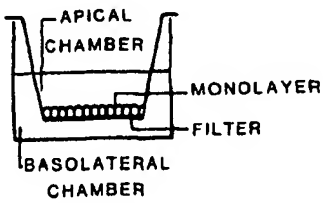

Figure 1—Diagram of apparatus used to grow cells.

The integrity of the monolayers was checked at the end of each experiment by measurement of the transmembrane resistance. The monolayers were then washed five times with PBS and the radioactivity of the filters was determined. The results were corrected for dilution and expressed as the concentration (C) at time t.

All rate constants were obtained under 'sink' conditions (i.e., before >10% of the drug had been transported) from the linear drug appearance curves in the receiving chambers and were expressed as mol · L^{-1} · h^{-1}. (No disappearance slopes could be obtained for the hydrophilic drugs that were transported slowly across the monolayers.) By the use of 'sink' conditions, the influence of drug diffusing back from the receiving to the donor chamber could be minimized. The regression coefficients (r^2) obtained from the linear curve fits were generally 0.98–1.00.

The apparent permeability coefficient (P_{app}) was determined according to the following equation:[13]

$$P_{app} = \frac{\Delta Q}{\Delta t 60 A C_o} \text{ (cm · s}^{-1}\text{)} \quad (1)$$

where $\Delta Q/\Delta t$ is the permeability rate (μg · min^{-1}), C_o is the initial concentration in the donor chamber (μg · mL^{-1}), and A is the surface area of the membrane (cm²).

Results

Characterization of the Cells—*Choice of Supports*—In previous studies, Caco-2 cells have been cultivated on membrane filters made of mixed cellulose esters or on collagen-coated polycarbonate filters.[5,7] The mixed cellulose ester supports were unsuitable in this study since they adsorbed significant amounts of the radiolabeled drugs (Table I). Polycarbonate supports were found to be a better alternative. Thus, 1.0 ± 0.3% of a 1 × 10^{-4} M solution of metoprolol was found in the polycarbonate filters after 1 h incubation at 37 °C, while the corresponding figure for the supports based on cellulose was 29.8 ± 0.6%. The characteristics of Caco-2 monolayers grown on polycarbonate supports were comparable with those cultivated on the cellulose based supports (Table II). The subsequent studies were performed on polycarbonate supports.

Cell Growth and Transepithelial Resistance—The cell density and transmembrane resistance of the Caco-2 cells was followed for 30 days (Figures 2–4). Both of these parameters increased in parallel during the first 9 days of cultivation (Figure 2). At this time the cell growth plateaued as did the resistance. Thus, a cell density of ~0.9 × 10^6 cells/cm² and

Table I—Absorption of Drugs to Different Cell Culture Supports[a]

Drug	Concentration, M	log D	% Absorbed	
			Polycarbonate	Cellulose
Propranolol	1×10^{-3}	1.19	23.4 ± 0.03	52.2 ± 1.5
	1×10^{-4}		0.9 ± 0.2	41.3 ± 4.2
Metoprolol	1×10^{-3}	−0.26	2.4 ± 0.1	35.2 ± 3.3
	1×10^{-4}		1.0 ± 0.3	29.8 ± 0.6
Atenolol	1×10^{-3}	−2.14	0.08 ± 0.002	15.6 ± 0.6
	1×10^{-4}		0.05 ± 0.002	6.0 ± 0.4

[a] The filters were incubated with the radiolabeled drugs for 1 h at 37 °C, and washed (×5) with phosphate-buffered saline before counting in a liquid scintillation counter (n = 3; ±SD).

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Table II—Characterization of Caco-2 Monolayers Grown on Different Cell Culture Supports[a]

Characteristic	Polyacarbonate	Cellulose
Cells/cm^2 × 10^{-6}	0.91 ± 0.04 (3)	0.88 ± 0.06 (3)
Transmembrane resistance, ohms·cm^2	258 ± 40 (45)	275 ± 55 (31)
[^{14}C]PEG transport, % of dose/4 h	0.05 ± 0.03 (3)	0.02 ± 0.04 (3)

[a] The experiments were performed as described in the *Experimental Section*; the number of experiments is given within brackets, and the results are expressed as ±SD.

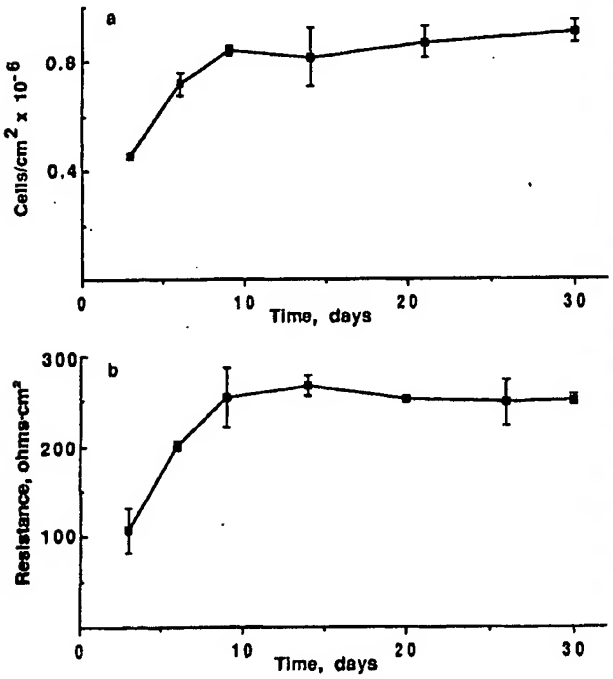
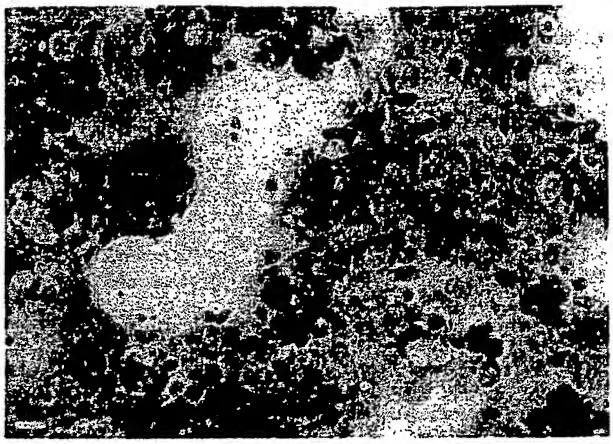


Figure 2—Cell density (a) and transmembrane resistance (b) as a function of time (n = 3; ±SD).

a transmembrane resistance of ~260 ohms·cm^2 was obtained. This resistance was higher than the 170 ohms·cm^2 recently reported for collagen-coated polycarbonate filters.[5] Photomicrographs of cells obtained 3 days after initiation of the cultures showed that although the cells were well spread over the surface of the filters, the cell number was too low to give confluent monolayers (Figure 3a). However, 9 days later, the cells formed a dense monolayer without intercellular spaces (Figure 3b). It was noted that the cells appeared to be compressed since each cell nucleus occupied a smaller surface area than in Figure 3a.

The distribution of transmembrane resistance for 103 monolayers having an age of 10–30 days is presented in Figure 4. The distribution is wider than could be expected from Figure 2. This can be explained by the fact that the transmembrane resistance varied more between different batches of the Caco-2 cells than within a single batch. Literature values of transepithelial resistances of rabbit and rat intestine have been included in Figure 4. The Caco-2 monolayers seem to have an electrical permeability that is similar to that of colonic epithelium.

Macromolecular Transport—Only very small amounts of ^{14}C-labeled polyethyleneglycol$_{4000}$ were transported across the epithelial cells (Figure 5). The insert in Figure 5 shows



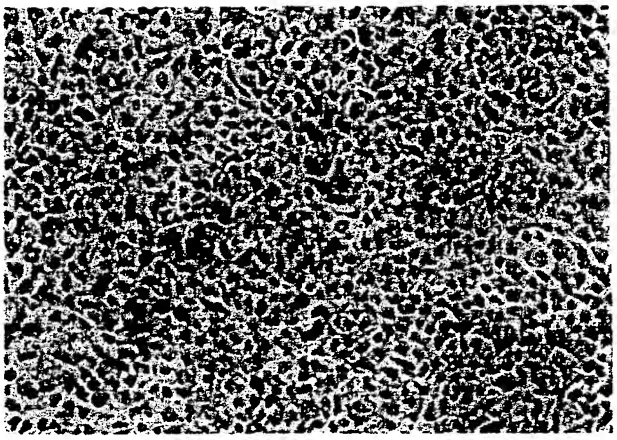


Figure 3—Photomicrographs of Caco-2 cells on filters 3 (top picture) or 14 days (bottom picture) after initiation of the cell cultures. The scale indicates 20 μm.

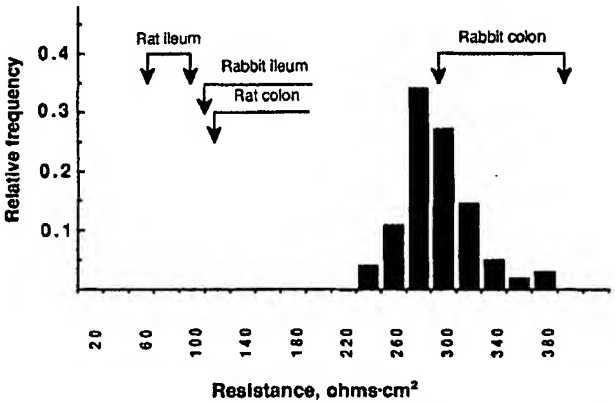


Figure 4—Distribution of transmembrane resistance for 103 monolayers of an age of 10–30 days. The resistance values of rat and rabbit intestines were taken from refs 36 and 37.

that monolayers that had ages of 9 and 24 days were equally impermeable to the macromolecule. When the transported radioactive material was gel filtered on a polyethyleneglycol-saturated Sephadex G-10 column, it had an elution volume similar to that of the low molecular weight fraction. Thus, the

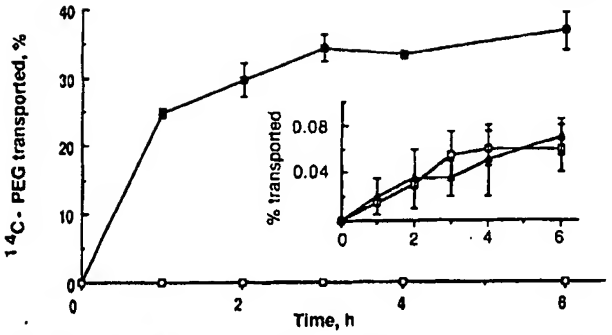


Figure 5—Transport of [^{14}C]polyethyleneglycol over empty polycarbonate filters (filled squares) or Caco-2 monolayers (open squares, triangles). The monolayers were 9 (triangles) or 24 days (open squares) old. The insert shows the same experiment in another scale (n = 3; ±SD).

fraction of the polyethyleneglycol that was transported over the monolayers had a molecular weight that was lower than that of the main fraction, which eluted with the high molecular weight fraction. Thus, the amount of high molecular weight material that was transported across the cells was lower than the small amounts shown in Figure 5.

Effect of Drugs on the Monolayers—The effects of different concentrations of the β-blockers on the integrity of the monolayers were studied for 6 h. No significant effects on transmembrane resistance, cell number, and polyethyleneglycol transport could be observed over a 10 000-fold range in metoprolol concentration (Figure 6). These results were verified in the transmission electron microscope (data not shown). Similar results were obtained for the other drugs.

Drug Transport Studies—The transport of metoprolol from the apical (luminal) to the basolateral side of the cells is presented in Figure 7. It is obvious that the curves follow first-order kinetics. It was not possible to calculate accurate rate constants (and apparent permeability coefficients) from these curves since the influence of drug diffusing back from the receiving to the donor chamber was significant at later stages. Instead, the rate constants were calculated from the initial straight slopes that were obtained under 'sink' conditions (see the insert in Figure 7).

No large differences in the transport rate of metoprolol were observed over a 10 000-fold concentration range (Figure 8a). Similar results were obtained for atenolol and propranolol. The exception is a small decrease in disappearance rate and a correspondingly small increase in appearance rate at the highest concentration. Thus, at a concentration of 1×10^{-4} M, the relative amount of drug that was interacting with binding sites on or within the cells or with the polycarbonate filters was too small to have an influence on the profiles of the curves (c.f., Table I). No differences in the transport rate could be observed if the drug was allowed to diffuse in the opposite direction (i.e., from the basolateral to the apical side; Figure 8b).

The transepithelial transport of six different β-blockers was studied (Figure 9). The concentration of the drugs was 1×10^{-4} M since the more lipophilic drugs were distributed into the filter/monolayer at lower concentrations (Table I and Figure 8A). The compounds could clearly be divided into two groups, those that rapidly diffused across the monolayers (propranolol, alprenolol, metoprolol) and those that were slowly transported (H216/44, practolol, atenolol).

A plot of the logarithm of the apparent permeability coefficient (P_{app}) against log D for the same six β-blockers is shown in Figure 10. The curve appears to have a sigmoidal shape and shows a plateau for the lipophilic drugs at a log D value of −0.5–1.0. The curve starts to level off towards a second lower plateau at a log D value of about −1–−2. The exception is the experimental compound H216/44. This relatively lipophilic drug was transported slowly across the Caco-2 cells (Table III and Figure 10).

The experimental compound H216/44 has a higher molecular weight (MW 480) than the other β-blocking agents (MW 249–260). Thus, the diffusion coefficient (as calculated from the Stokes–Einstein equation) was found to be ~020% lower for H216/44 as compared with the other drugs. A corresponding correction of the apparent permeability coefficient could therefore be introduced in order to obtain a more correct comparison with the other drugs in Figure 10. However, the

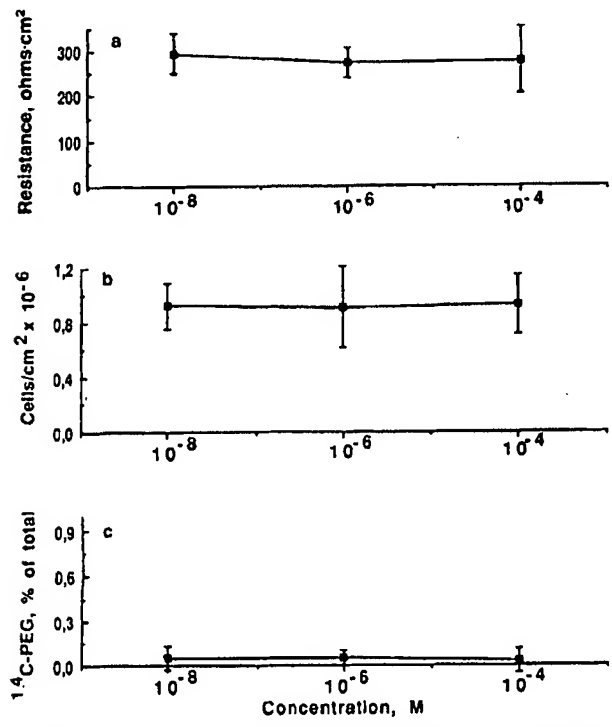


Figure 6—Transmembrane resistance (a), cell density (b), and polyethyleneglycol transport over Caco-2 monolayers (c) after treatment with different concentrations of metoprolol for 6 h (n = 3; ±SD).

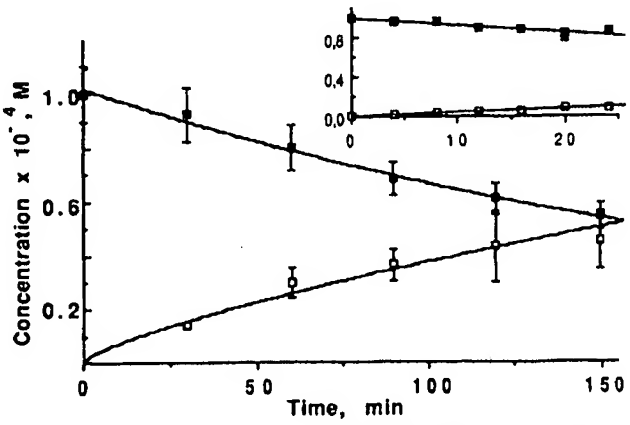


Figure 7—Transport of metoprolol (initial concentration 1×10^{-4} M) over Caco-2 monolayers. The insert shows a similar experiment performed under a shorter time period (i.e., under 'sink' conditions; n = 3; the error bars show the 95% confidence limits).

Table III—Distribution and Permeability Coefficients

Drug	log *D*	$P_{app} \times 10^6$, cm/s[a]
Propranolol	1.19	41.91 ± 4.31
Alprenolol	1.00	40.48 ± 1.22
Metoprolol	−0.28	26.95 ± 0.71
H216/44	−0.097	0.916 ± 0.15
Practolol	−1.40	0.898 ± 0.05
Atenolol	−2.14	0.203 ± 0.004

[a] P_{app} were calculated from the formula given in the *Experimental Section*.

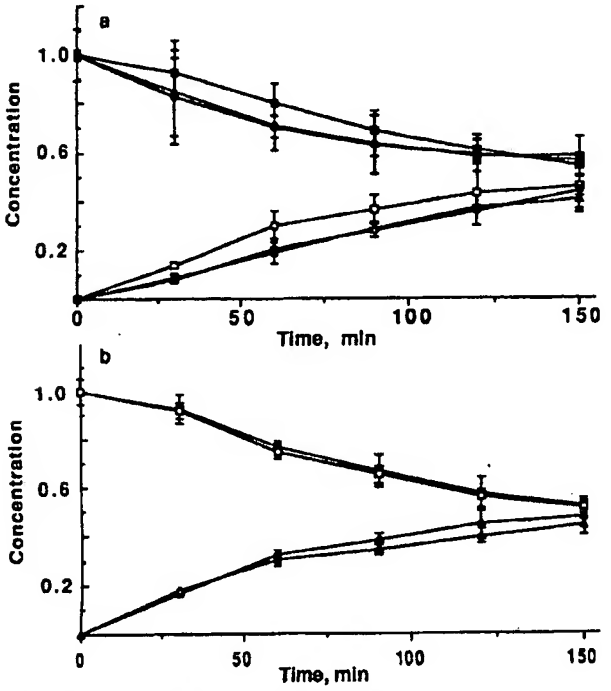


Figure 8—(a) Transport of different concentrations of metoprolol from the apical to the basolateral side of Caco-2 monolayers. The initial concentrations were 10^{-3} M (△, ▲), 10^{-5} M (◇, ◆), and 10^{-4} M (□, ■). (b) Transport of metoprolol (10^{-3} M) from the apical to basolateral (filled symbols) and from the basolateral to the apical side (open symbols; n = 3; ±SD).

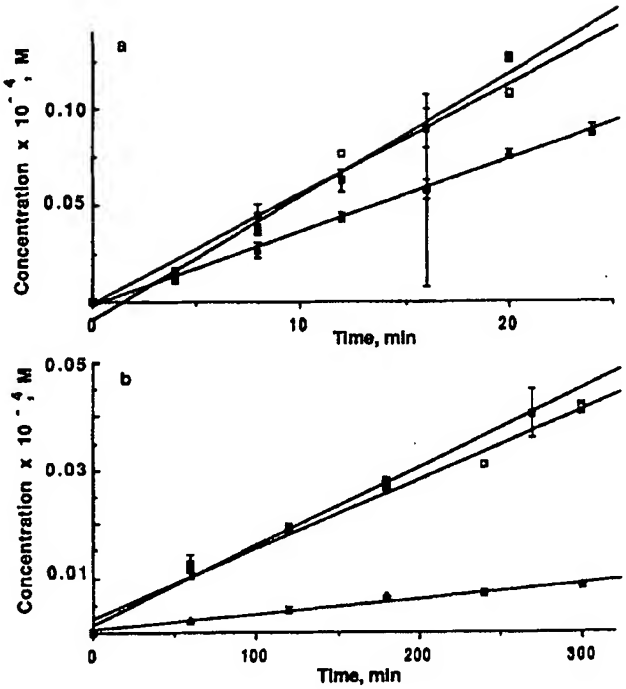


Figure 9—(a)Transport of propranolol (■), alprenolol (□), and metoprolol (△), and (b) H216/44 (■), practolol (□), and atenolol (△) over Caco-2 monolayers. All drugs were added to the apical chamber at a concentration of 1×10^{-4} M (n = 3; ±SD).

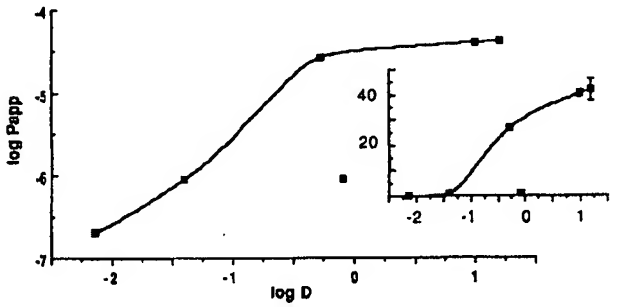


Figure 10—Apparent permeability coefficients as a function of the distribution coefficients for six β-blockers. The insert shows the same graph in a lin–log scale.

influence of the molecular weight was small and, consequently, no correction was made in Figure 10.

In Table IV, a comparison is made between the experimental data obtained in this study with literature data obtained in a rat in situ model.[18] In the latter, the drugs were introduced into an isolated ileal loop and the disappearance of the drugs from the lumen was followed.

The drug disappearance curves obtained in the rat model were exponential and followed first-order kinetics.[18] In comparison, the drug appearance curves in the Caco-2 model were linear. The rate constants of the two models could therefore not be directly compared. Instead, the times when 10% of the drugs had been transported ($t_{0.1}$) were calculated from the respective rate constants. At this time (i.e., under 'sink' conditions), the initial slopes of the two models were comparable.

Only relative comparisons can be performed between the Caco-2 model and rat small intestine since the rate constants were unrelated to the respective surface areas.[18] However, an approximate calculation of the surface area of the rat intestinal segment can be done by use of literature values of the radius of rat small intestine under similar conditions (i.e., 0.18 cm[19,20]), and the length of the intestinal segment used by Taylor et al. which was 7.5 cm.[18] This gives a surface area of 8.5 cm^2. If this value is divided by the surface area of the Caco-2 model (4.71 cm^2), a factor of 1.8 is obtained. A factor of 7–14 for the expected increase in surface area obtained by the mucosal villi[21] can also be introduced. This gives a surface area of the rat model of 59–118 cm^2. If this value is divided by the surface area of the Caco-2 model, a factor of 18.5 (12–25) is obtained. (It should be noted that the real surface area is ~25 times larger due to the contribution by microvilli,[22] but this factor can be excluded from the calculations since microvilli are also present on the Caco-2 cells.)

If the ratios between the $t_{0.1}$ values of the two models are multiplied with this factor, ratios of close to one will be obtained for metoprolol, alprenolol, propranolol, and practolol. Thus, after normalization of the surface areas, the transport rates of four out of five compounds were similar in

Table IV—Comparison of $t_{0.1}$ Values from the Caco-2 Model and Rat Ileum[a]

Drug	$K_{app\ Caco\text{-}2} \times 10^{-4}$, mol · L^{-1} · h^{-1}	$-K_{dis\ rat\ ileum}$, h^{-1}	$t_{0.1\ Caco\text{-}2}$, h	$t_{0.1\ rat\ ileum}$, h	Ratio[b]
Propranolol	0.348 ± 0.036	4.11 ± 0.84	0.287	0.0256	0.161
Alprenolol	0.342 ± 0.010	3.94 ± 0.25	0.292	0.0267	0.164
Metoprolol	0.228 ± 0.006	2.86 ± 0.28	0.439	0.0368	0.151
H216/44	0.00775 ± 0.0008	n.d.	12.90	n.d.	—
Practolol	0.00760 ± 0.0006	0.26 ± 0.16	13.16	0.405	0.055
Atenolol	0.00171 ± 0.00004	0.22 ± 0.16	58.47	0.479	0.015

[a] Rate constants (Caco-2) were calculated from the drug appearance slopes of individual experiments (n = 3; ±SD); rate constants (rat ileum) were taken from ref 18 (n = 8–12; SD were calculated from the SEM values, assuming n = 10); the $t_{0.1}$ values were calculated from the rate constants, see *Results*; n.d. = not determined. [b] The ratios were obtained from $t_{0.1\ Caco\text{-}2}$ divided by ($t_{0.1\ rat\ ileum} \times 1.8$); see *Results*.

the two models, while the most hydrophilic drug, atenolol, was transported over the colonic Caco-2 cells at a rate six times slower than that obtained in rat small intestine. A reservation to the calculations above is that the closeness of adjacent villi and the inaccessibility of the lower parts of the villi and crypts may reduce the available surface area of the rat ileum. This means that the permeability of the Caco-2 model may be lower than that of the rat ileum. However, the ratios for the transport of the drugs in the two models will still be similar for four out of five drugs (Table IV).

Discussion

Recently, Caco-2 cells have been characterized on collagen-coated polycarbonate filters.[5] In this study, the Caco-2 cells were grown on uncoated polycarbonate filters. It was therefore important to confirm that the Caco-2 cells also formed continuous monolayers with this simplified procedure. The findings that the electrical resistance remained at a constant maximal level and that the cells were impermeable to a macromolecular marker between days 10 and 30 in culture indicated that the monolayers could be used for passive drug diffusion experiments for at least 20 days. This was confirmed in studies with monolayers of different ages where no differences in the drug transport parameters were observed. One explanation to the requirement of collagen in the paper by Hidalgo et al.[5] may be that they used polycarbonate filters with a mean pore diameter of 3.0 μm as compared with the 0.45-μm diameter used in this study. It is likely that the formation of intact monolayers was facilitated if the collagen covered these relatively large pores. A recent preliminary report supports the finding that Caco-2 cells form continuous monolayers on uncoated polycarbonate filters.[23]

However, other differentiation markers, such as different brush border hydrolases, are constantly increasing in concentration over a time period of at least 20 days in culture.[24] Thus, complementary assays to those used in this study will be needed to characterize the Caco-2 cells if, for instance, intestinal drug metabolism is to be studied.

The findings in this study indicate that the β-blockers were transported by passive diffusion over the Caco-2 cells. Thus, the transport was not saturable and the transport rate from the luminal to the basolateral side of the monolayers was the same as that in the opposite direction. This is not in agreement with the postulated net secretion of weak bases into the intestinal lumen.[1] One explanation for the different results could be that the range of concentrations used in the present study were too high and therefore obscured a possible active transport mechanism which may be dominant at lower concentrations. This hypothesis is less likely since several active transport systems that have been identified in the Caco-2 system can be detected at the concentrations used in this study.[7,9–11] A more likely explanation is that the net transport of weak bases is regulated by extracellular factors. Thus, the net serosal-to-mucosal transport has been suggested to depend on differences in extracellular pH rather than on the epithelial monolayer itself.[25] The difference between the Caco-2 and whole tissue models can therefore be explained since in this study, all experiments were performed at a serosal and/or mucosal pH of 7.3.

There are mainly three barriers to the passive diffusion of drugs over the intestinal epithelium.[24] Adjacent to the apical surface of the epithelium there is an aqueous boundary layer. This so-called 'unstirred' water layer has been shown to be rate limiting for highly lipophilic compounds that have high membrane permeabilities.[19,20,26] (For more hydrophilic compounds, the membrane permeability is rate limiting.) The apparent permeability coefficients of the more lipophilic drugs may therefore be biased by the presence of an 'unstirred' water layer.

The epithelial monolayer is comprised of the other two major permeability barriers: the lipophilic cell membranes and the intercellular junctions between the cells. The intercellular spaces are sealed by tight junctions which reduce their pore radius to a few Angstrom units.[21,27] In addition, the area of the intercellular spaces is negligible compared with the total surface area of the epithelium.[28] Thus, the contribution of this paracellular pathway to the total permeability of the epithelial monolayer is only significant for drugs that are transported slowly across the cell membrane (e.g., hydrophilic compounds that have very low membrane partition coefficients). However, most drugs are relatively lipophilic and will partition rapidly into the cell membranes. The results in the Caco-2 model are in agreement with this statement. In general, the more lipophilic drugs were transported rapidly across the cells, while the hydrophilic compounds had permeability coefficients that were 46–206 times lower than that of the most lipophilic drug (propranolol). However, H216/44 (which is more lipophilic than metoprolol) was transported at a relatively slow rate across the monolayers. The reason for this is currently unknown, but may be related to the bulky chemical structure of this compound. Another lipophilic β-blocking agent, acebutolol, has also been reported to be absorbed at an unexpectedly slow rate across intestinal epithelium.[18]

The subepithelial barriers, such as the basement membrane, lamina propria, and the capillary wall, are not generally considered to be rate limiting for the absorption of small drug molecules.[1] Thus, with the exception of mucin which can bind and thereby decrease the absorption of some drugs, the Caco-2 model seems to contain the three major permeability barriers to the absorption of drugs; that is, the 'unstirred' water layer, the junctional complex (including tight junctions) between the cells, and the cell membranes.[5–7] Drugs that are metabolized by intestinal brush border enzymes have not been considered in this paper. It is likely that the Caco-2 cells will also be useful for metabolism studies since they have many of the brush border enzymes of small intestinal mucosa in quantities similar to those found in vivo.[24]

A good correlation was found between the results in the Caco-2 model and those obtained with the same β-blockers in a rat in situ model.[18] The ratios between the $t_{0.1}$ values in the different models were similar for four out of five drugs. The exception was atenolol which was absorbed at a relatively slower rate in the Caco-2 model. The transepithelial uptake of hydrophilic markers in rat and hamster intestinal mucosa has recently been shown to correlate with paracellular permeability, measured as transmembrane resistance.[29] Thus, it seems reasonable that the slower transport of the hydrophilic drug atenolol in the Caco-2 model is a consequence of the lower paracellular permeability in this model as compared with the rat ileum. This statement is also supported by our recent finding that the reversible opening of the tight junctions in the Caco-2 model results in increased transport rates of the hydrophilic but not the hydrophobic β-blockers.[30] Moreover, studies on metoprolol and oxprenolol (a β-blocker with a log *D* similar to that of metoprolol) in humans show that these hydrophobic drugs are absorbed at the same rates in jejunum, ileum, and colon (i.e., over the entire intestine[31–33]). However, it should be emphasized that measurements of transmembrane resistance are relatively insensitive to small changes in epithelial permeability.[34] Other permeability markers, such as hydrophilic molecules of various molecular weights, should also be used.

The Caco-2 model had a good reproducibility. In general, triplicate samples gave standard deviations of <10%. This was also the case when very small amounts of the drugs were transported. By comparison, in the rat model, 8–12 intestinal segments were used. Still, the standard deviations for the rate constants of the more hydrophilic compounds were sometimes ~50%.[18]

The findings in this paper indicate that the Caco-2 model may be a useful alternative to animal models for the study of intestinal absorption of drugs. The morphological and biochemical properties of the Caco-2 cells are more similar to those of the small intestinal villus cells than to colonic cells.[6–7] However, the permeability is similar to that of colonic epithelium. Several drugs (including peptides) that are absorbed in the small intestine but not in the colon have very low permeability coefficients in the Caco-2 model.[35] This indicates that the model could be used to differentiate those drugs that are not well absorbed in the large intestine. Knowledge of these factors could be usefully applied in the design of formulations for controlled-release systems. In addition, the model can be used to determine 'general' intestinal permeability coefficients for drugs that are transported mainly by the transcellular route since these drugs can be expected to be absorbed at the same rate throughout the whole intestine.[31–33]

Good correlation between rat ileal absorption and the Caco-2 model was obtained for four out of five drugs. These four drugs ranged 400-fold in lipophilicity which shows that the model is suitable not only for lipophilic, but also for relatively hydrophilic drugs. However, there is a need for other cell lines with properties that also model the absorption profiles of very hydrophilic drugs such as atenolol. The transport of other drugs that have different chemical and physical properties must also be studied before a more complete evaluation of the model can be made.

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